EXHIBIT C

Thrombin regulation of endothelin-1 gene in isolated human pulmonary endothelial cells

CATHERINE L. GOLDEN,[1] HARRY S. NICK,[2] AND GARY A. VISNER[1,3]
Departments of [3]Pediatrics, [1]Biochemistry and Molecular Biology, and [2]Neuroscience, University of Florida, Gainesville, Florida 32610

Golden, Catherine L., Harry S. Nick, and Gary A. Visner. Thrombin regulation of endothelin-1 gene in isolated human pulmonary endothelial cells. *Am. J. Physiol.* 274 (*Lung Cell. Mol. Physiol.* 18): L854–L863, 1998.—Endothelin (ET)-1 is a potent vasoconstrictor elicited from endothelial cells in response to a variety of stimuli and an important mediator for a variety of vascular diseases including pulmonary hypertension. In this paper, we describe the molecular regulation of the ET-1 gene in response to a vasoactive mediator, thrombin, in human pulmonary endothelial cells. Thrombin induces preproET-1 mRNA through a transcriptionally dependent mechanism, with a peak induction after 1 h of exposure. Analysis of chromatin structure identified several DNase I-hypersensitive regions under both basal and thrombin-stimulated conditions that reside in the 5′-promoter region, indicating that the ET-1 promoter is a constitutive promoter. Deletion analysis was employed as a functional assay to identify regions of the ET-1 promoter that are important in transcriptional regulation. We found that sites between −141 and −378 bp are essential for basal activity and that those between −378 and −484 bp are essential for thrombin-stimulated activity. However, full expression under both conditions required an element(s) within −952 bp.

chromatin studies; deletion analysis; pulmonary vascular disease

IT HAS BEEN well established that the endothelium is vital in the regulation of reactivity in vascular tissues via release of endothelium-derived factors that act on adjacent smooth muscle cells. One such factor produced by vascular endothelial cells is the potent vasoconstrictive peptide endothelin (ET)-1, which is regulated by a number of inflammatory and vasoactive mediators (11, 15, 20, 36). Regulation of ET-1 secretion by these mediators is most likely critical in maintenance of vascular tone in certain pathophysiological situations.

Our laboratory is particularly interested in the mechanisms present in pulmonary disease states, especially pulmonary hypertension and adult respiratory distress syndrome. Mounting evidence implicates ET-1 as a mediator of these pathologies (8, 10, 16, 30, 31). Therefore, studies aimed at understanding the molecular mechanisms underlying ET-1 gene expression in human pulmonary endothelial cells may lead to data relevant to treatment of these human pathologies.

The mature 21-amino acid ET-1 peptide is derived by proteolytic processing from preproET-1, a 212-amino acid precursor. Understanding ET-1 gene expression under constitutive as well as stimulated conditions is imperative for our understanding of the role of ET-1 in pulmonary and vascular disease. Functional assays have been used by two different groups to provide a fairly detailed picture of the basal regulation of ET-1 in bovine endothelial cells (6, 14, 17, 18, 34) and in COS cells (1). In addition, a very limited analysis of stimulus-induced ET-1 expression, using functional studies, has been conducted (19, 23). However, there are limitations to these previous investigations, as these studies have been conducted in a heterologous system whereby regulation of the human ET-1 gene was assessed in bovine or porcine aortic endothelial cells, COS cells, HeLa cells, or NIH/3T3 cells, in some cases with variable results. On the basis of reports of species- and tissue-dependent differences in ET-1 gene expression (26) and reports that endothelial cells derived from different species and tissues can express dramatic variations in metabolic and enzymatic properties (3, 7, 25, 29), we believe that investigation of human gene regulation should be conducted in human tissue-derived cells relevant to disease. Additionally, we need a better understanding of the regulation of this gene, not only under basal conditions but also under stimulated conditions that mimic the vascular environment to which endothelial cells are exposed during disease states.

In this work, we provide a detailed analysis of the expression and regulation of the human ET-1 gene in human pulmonary endothelial cells. The aim of these studies was to investigate the effect of thrombin, a physiological mediator potentially involved in the pathogenesis of pulmonary vascular disease, on the molecular regulation of ET-1.

METHODS

Cell culture. Isolation of human pulmonary arterial (HPA) endothelial cells was performed essentially as described (32), with some modifications. Cells were obtained from HPA segments from heart transplant donors by collagenase (Sigma, St. Louis, MO) treatment and scraping of the endothelium. The scraped cells were placed in endothelial basal medium (EBM; Clonetics, San Diego, CA), pelleted, resuspended in EBM plus 10% fetal bovine serum (FBS), and plated in cell culture flasks. The cells were incubated at 37°C in room air containing 5% CO_2, and the medium was changed within 24 h and then every two days until confluence. Once the cells reached confluence, they were trypsinized using 0.025% trypsin and 0.02 mM EDTA and passed at a ratio of 1:3 or 1:4 in EBM plus 10% FBS. Human pulmonary microvascular (HMV) endothelial cells were obtained commercially from Clonetics. These cells were passed under the same conditions as the HPA cells. All cells used for the following investigations were at low passage, with no studies performed above *passage 11.*

Northern analysis. HPA and HMV endothelial cells were grown to confluence on 100-mm cell culture dishes. ET-1 mRNA levels were assessed under both basal and thrombin-stimulated conditions. Cells were exposed to bovine thrombin (10 U/ml; Sigma) or to purified bovine thrombin (ICN) for a period of 15 min to 4 h. Similar results were obtained with

1040-0605/98 $5.00 Copyright © 1998 the American Physiological Society

both products; therefore, the majority of the experiments were performed with the Sigma preparation. Each treated plate had a corresponding control plate from the same cell line and passage number. Inhibitor studies were performed to better characterize the regulatory mechanisms involved in ET-1 gene expression at the transcriptional level. Cells were cotreated with thrombin (10 U/ml) and either actinomycin D (4 μM; Sigma), an RNA synthesis inhibitor, or cycloheximide (20 μM; Sigma), a protein synthesis inhibitor.

Total cellular RNA was isolated according to the procedures presented by Chomczynski and Sacchi (4), and Northern analysis was performed as described previously (11), with human ET-1 or cathepsin B cDNAs used as probes. The human ET-1 cDNA was isolated by our laboratory as previously described (32). The cathepsin B cDNA was a gift from S. J. Chan (Univ. of Chicago) and was used as an internal control to correct for any variations in RNA loading. Northern blots were exposed to autoradiography with an intensifying screen at −85°C and were analyzed by densitometry using a BioImage video-image processor.

Preparation of permeabilized cells, DNase I digestion, and DNA isolation. Six 150-mm cell culture dishes of HPA endothelial cells grown to confluence were placed in EBM containing 1% FBS. The next day, three plates were kept as controls, receiving no treatment, and three plates were treated with 10 U/ml of thrombin for 20–30 min. These were washed and trypsinized, and 5 ml of cold medium were added to each plate. The cells from each set were scraped, collected, and centrifuged at 600 *g*, 4°C, for 5 min and resuspended in 4.5 ml of *solution A* (150 mM sucrose, 80 mM KCl, 35 mM HEPES, pH 7.4, 5 mM potassium phosphate, and 5 mM $MgCl_2$). Then 1.5 ml of 0.2% L-α-lysolecithin in 60 mM KCl, 15 mM NaCl, 60 mM Tris, pH 7.8, and 0.25 M sucrose were added; this mixture was incubated for 2 min on ice; and 30 ml of *solution A* were added to stop the reaction. The cell suspension was spun again and resuspended in 1,200–1,500 μl of *solution A*, yielding 2×10^7 cells/suspension. Cells from each suspension were then split into 4–5 aliquots of 300 μl each. The cell suspensions were treated with increasing concentrations of DNase I (2.5 mg/ml of stock; Worthington Biochemical, Freehold, NJ) at 37°C for 4 min. Lysis buffer (100 mM EDTA, 1% SDS, and 5 μg/ml of proteinase K) was added to each sample and rocked for 3 h at 55°C. Genomic DNA was extracted by standard phenol-chloroform purification procedures. The DNA was treated with RNase A (50 μg/ml; Sigma) followed by ethanol precipitation and resuspended in water.

Southern analysis. Twenty micrograms of DNA were digested and size fractionated on an agarose gel, and Southern analysis was performed (27). The DNA was electrotransferred to an uncharged nylon membrane (Zetabind, Cuno, Meriden, CT) and cross-linked by ultraviolet irradiation. The method of indirect end labeling was used to map the positions of hypersensitive sites (35). Both high- and low-resolution chromatin structure was evaluated. For low-resolution studies, DNA was size fractionated on a standard 1% agarose gel, and high-resolution analysis was performed with the use of MetaPhor (FMC BioProducts, Rockland, ME) agarose gel system, which more accurately resolves smaller fragments than standard agarose.

A full-length ET-1 genomic clone was obtained commercially (Genome Systems, St. Louis, MO) by screening a human P1 bacteriophage library for clones positive for the 5′- and 3′-ends of the ET-1 gene. Subclones of the ET-1 gene in pUC19 were constructed for use in making probes and promoter deletion fragments for chromatin structure and promoter deletion analysis. Probes for DNase I analysis were created either by isolation of restricted fragments from ET-1 gene subclones using gratuitous restriction sites or by amplification of the desired fragment using PCR primers. Labeling of probes by [^{32}P]dATP was accomplished by random-primer extension (GIBCO BRL).

Plasmid constructions. Portions of the ET-1 promoter were cloned into a promoterless human growth hormone (hGH) reporter vector, pØGH (Nichols Institute, San Juan Capistrano, CA), for use in promoter deletion analysis. The largest ET-1-pØGH reporter plasmids were created by cloning portions of the ET-1 gene using gratuitous restriction enzyme sites and with ligation into the polylinker of pØGH. These plasmids contain 8, 5.2, and 2.4 kb of ET-1 sequence 5′ to the transcriptional start site. Each of these plasmids has a common 3′ site located 166 bp 3′ to the transcriptional start site, created by a *Bgl* II restriction enzyme site at this position. The 8- and 5.2-kb ET-1-promoter fragments were created by cleavage of the ET-1 gene with *Bgl* II and *Xba* I, respectively. The desired fragments were cloned into the *Bam*H I site or the *Xba* I and *Bam*H I sites, respectively, of pØGH. Similarly, the 2.4-kb ET-1 fragment, which is flanked by an *Xho* I site at −2.4 kb, was created by liberating the desired fragment from a second subclone of the ET-1 gene by digestion of this clone with *Xba* I (in the polylinker of pUC19) and *Bgl* II (at +166 bp of ET-1). This product was directionally cloned into the *Xba* I and *Bam*H I sites of pØGH. All positive clones were confirmed by restriction mapping and sequence analysis.

The −628-bp construct was created by PCR amplification using a 5′ primer containing an *Xba* I linker site at the 5′-end and was complementary to the ET-1 sequence at positions −628 to −608. The 3′ primer was a 20 mer that spanned a region that included the *Bgl* II site at position +166 in exon 1. After amplification, the fragment was restricted with *Xba* I and *Bam*H I and directionally cloned into the *Xba* I and *Bam*H I sites of pØGH.

The other six plasmids used for this work were obtained from the laboratory of Dr. David Wilson (Children's Hospital, St. Louis, MO) (34). These plasmids were sequenced by a fluorescence-based cycle-sequencing reaction to determine their correct size and location in the ET-1 gene. These reactions were conducted on high-purity DNA preparations using an ABI Prism dye terminator cycle-sequencing core kit with Ampli*Taq* DNA polymerase on an ABI 373A DNA sequencer. These plasmids contain various size fragments of the 5′-promoter region of ET-1 and a common 3′-end at the *Bgl* II site within exon I. The ET-1-promoter regions contained in these plasmids are of the following sizes: −1,376, −952, −484, −378, −141, and −88 bp.

Transient transfection methods. HPA or HMV endothelial cells were grown to ~70% confluence in 150-mm cell culture dishes. Plasmid DNA (8.1 μg) was brought to 80-μl volume in Tris-buffered saline and combined with 224 μl of a 10 mg/ml stock solution of DEAE-dextran (Sigma). The DNA-DEAE-dextran solution was added to cells in 10.8 ml of EBM supplemented with 10% NuSerum (Collaborative Biomedical Products, Bedford, MA) for a final DEAE-dextran concentration of 200 μg/ml. To inactivate the lysosomes of the cell and thereby prevent degradation of the transfected DNA, chloroquine diphosphate (Sigma) was added to the media at a final concentration of 75 μM. Cells were incubated for 4 h at 37°C in room air with 5% CO_2. Media were poured off, and 13.5 ml of 10% DMSO (Sigma) in PBS were added to each plate for 1.5 min. The cells were washed in PBS, EBM plus 10% FBS was added, and cells were incubated at 37°C. The next day, cells were passed to 4 × 100-mm culture dishes (27). On *day 3* after transfection, the medium was changed to EBM containing 1% FBS, and several hours later, two of the four plates were kept

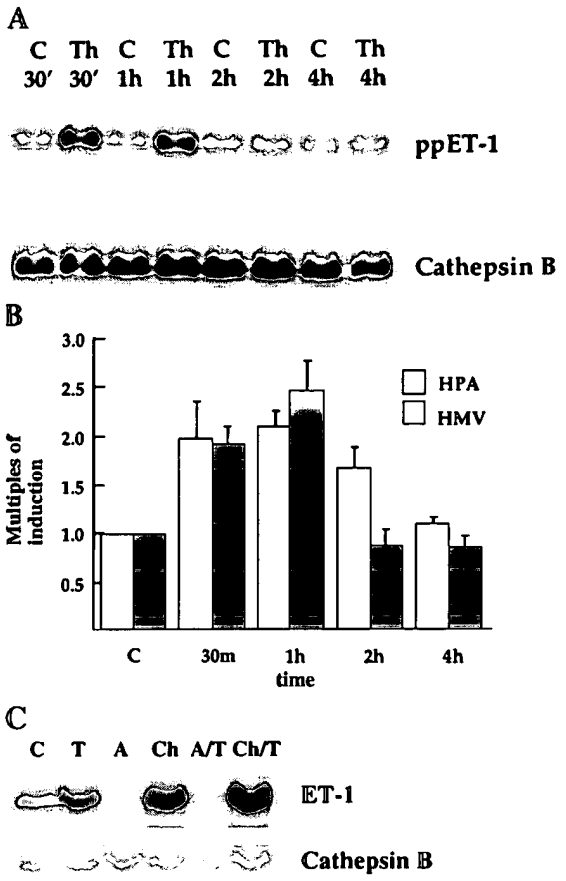

Fig. 1. Endothelin (ET)-1 mRNA expression in human pulmonary microvascular (HMV) versus human pulmonary arterial (HPA) endothelial cells. ET-1 mRNA levels were determined by Northern analysis on HMV and HPA endothelial cells grown to confluence. Total RNA was isolated, and 20 μg were fractionated on a 1% agarose-formaldehyde-MOPS buffer gel and electrotransferred to a noncharged nylon membrane. Human ET-1 mRNA levels were determined by hybridization of membrane with a radiolabeled human ET-1 cDNA. Hybridization with a radiolabeled rat cathepsin B cDNA was conducted as an internal control. *A*: a representative blot of HMV cells treated with thrombin (Th; 10 U/ml) for 30 min (30') and 1, 2, and 4 h. At each time point, there were corresponding control (C) plates treated with vehicle. ppET-1, preproET-1. *B*: average multiples of induction of ET-1 mRNA levels in HMV vs. HPA endothelial cells under control and thrombin-stimulated conditions over time. All control values are set to 1, and multiples of induction are illustrated relative to control values. Data are means ± SE of 3–6 experiments. *C*: ET-1 mRNA levels in HPA endothelial cells cotreated with thrombin and RNA synthesis or protein synthesis inhibitors. Confluent HPA endothelial cells were exposed to thrombin (T), actinomycin D (A), cycloheximide (Ch), or a combination of these for 1 h.

as control plates and two were treated with thrombin (10 U/ml). Media were collected 72 h after treatment, and hGH levels were measured using a commercially available radioimmunoassay (Nichols Institute).

To evaluate plate-to-plate variability of transfection efficiency, a Southern analysis of DNA isolated from cells transfected with the ET-1 promoter-hGH plasmid was performed (22). At the termination of the experiment, the cells from control and stimulus-treated plates were lysed, and DNA was isolated and digested to generate a unique fragment. The DNA fragments were separated by size on an agarose gel and transferred to a nylon membrane that was hybridized to a probe within the promoter deletion fragment. Autoradiography was performed, and the relative amounts of plasmid were evaluated by densitometry.

Data analysis. Statistical comparisons were made for each vector individually to detect differences under stimulus versus control conditions. Values were calculated using a paired two-tailed *t*-test. All data are expressed as means ± SE, and significance was set at $P < 0.05$.

RESULTS

Detailed analysis of thrombin-stimulated ET-1 mRNA levels. Figure 1, *A* and *B*, illustrates ET-1 mRNA levels in HMV and HPA cells in response to thrombin stimulation over the course of 4 h. Figure 1*A* is a representative Northern blot from HMV cells, with nearly identical results for HPA cells. The graph in Fig. 1*B* illustrates a comparison of the multiples of induction of ET-1 mRNA for both cell lines. As illustrated, ET-1 mRNA levels are elevated over control values as early as 30 min after thrombin stimulation. After a peak induction at 1 h, ET-1 mRNA levels decline out to 4 h. Shown is a summary of three separate experiments for both cell lines.

To determine whether ET-1 mRNA induction by thrombin was a result of de novo transcriptional events, stability of ET-1 transcripts, or a combination of the two, cells were cotreated with thrombin and actinomycin D (a transcriptional inhibitor) or cycloheximide (a protein synthesis inhibitor). Shown in Fig. 1*C* is a representative result in HPA endothelial cells, and nearly identical results were seen in HMV cells (data not shown). As illustrated, exposure to actinomycin D alone caused a downregulation of ET-1 mRNA. This level is well below that of control values, which is indicative of the short half-life for ET-1 transcripts. Basal ET-1 mRNA half-life, as determined with actinomycin D treatment, is ~20 min (data not shown), which is similar to previous reports for other endothelial cells (15, 20). ET-1 mRNA induction in response to thrombin was blocked on coexposure with actinomycin D, indicating the requirement of a transcriptional event.

In contrast, cycloheximide exposure alone caused an ~2.5-fold induction of the message over basal levels. On cotreatment with cycloheximide and thrombin, a superinduction of ET-1 mRNA was noted over that of thrombin or cycloheximide treatment alone. This suggests that under basal and stimulated conditions, protein synthesis events may dampen ET-1 mRNA levels.

Promoter activity analysis. Our data, using actinomycin D to block transcription, indicated that ET-1 mRNA is induced in response to stimuli as a result of de novo transcriptional events. However, this does not prove that ET-1 itself has undergone de novo transcription. We have chosen to employ transient transfection of a promoter fragment of ET-1 fused to the hGH reporter gene to assay for de novo transcription of ET-1. An 8.0-kb fragment of the ET-1 promoter fused to the hGH reporter vector (pØGH) was introduced into both HPA and HMV endothelial cells and assayed for reporter protein expression. Results of this analysis are shown in Fig. 2, *A* and *B*. The 5' region of the ET-1 gene

possesses de novo transcriptional activity under basal conditions in both cell lines as evidenced by expression of the hGH reporter protein. No secreted hGH was detectable with the vector (pØGH) alone. Additionally, the ET-1 promoter confers increased transcriptional activity in response to thrombin stimulation, since hGH protein levels are induced over control values after stimulation. This response is seen for both HPA (Fig. 2*A*) and HMV (Fig. 2*B*) endothelial cells. A metallothionein promoter-driven hGH vector was also transfected as a control for thrombin-dependent changes of growth hormone, and thrombin was shown to have no differential effects compared with control conditions (data not shown).

Chromatin structure analysis. To provide evidence of potential binding sites for *trans*-acting factors on *cis*-acting DNA sequences, ET-1 gene chromatin structure was evaluated by mapping DNase I-hypersensitive sites surrounding the gene in HPA endothelial cells. The strategy for locating these sites involved first scanning the entire gene at low resolution from both the 5′- and the 3′-end. As illustrated in Fig. 3*A*, genomic DNA of basal and thrombin-stimulated HPA cells was cleaved with *Xho* I, which liberated a 10-kb fragment that encompassed the ET-1 gene. *Probe I*, which is 378 bp in size and abuts the *Xho* I site at the 3′-end of the gene, was employed to indirectly label this fragment and to scan the gene from the 3′ to 5′ perspective. When no DNase I was added (*lane 0*), the expected 10-kb fragment was detected, whereas with increasing concentrations of DNase I, a very strong hypersensitive site is evident in the promoter region just upstream of transcriptional initiation, with two additional sites near the 5′-end of exon V. At this level of resolution, there are no detectable differences in DNase I sensitivity between control and thrombin-stimulated conditions. Similar analysis of this region from the 5′-end toward the 3′-end using a probe that abuts the *Xho* I site at −2,459 bp showed identical results.

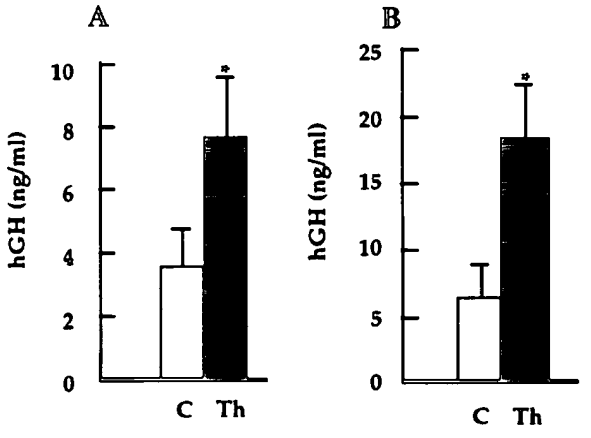

Fig. 2. Transient transfection of an 8.0-kb promoter fragment of ET-1 gene. An 8.0-kb promoter fragment of ET-1 gene was fused to a human growth hormone (hGH) reporter vector and introduced into HPA (*A*) and HMV (*B*) endothelial cells. Secreted hGH was assayed as a marker of transcriptional activity of ET-1 promoter under control and thrombin-stimulated conditions. * Significant difference between control and thrombin treatment ($P < 0.05$).

We then scanned the gene further upstream for hypersensitive sites with *probe II*, which abuts the *Bam*H I site at position +1,266 bp. Indirect end labeling with this probe allowed analysis from within the first intron to ~8 kb upstream from the transcriptional start site. As shown in Fig. 3*B*, with increasing concentrations of DNase I, a number of hypersensitive regions become apparent. The promoter region is especially populated with a number of prominent sites. Additional sites from those noted in Fig. 3*A* were identified within the first intron and first exon. Further upstream sites were observed at ~3.2 and 6.7 kb 5′ to the transcriptional initiation site. Again there is no difference in the position or the number of hypersensitive sites between control and thrombin-stimulated cells at this resolution.

To further detail the sites identified in the promoter region, a higher resolution analysis was performed using the MetaPhor (FMC BioProducts) agarose system, which allowed fractionation of smaller DNA fragments. In this analysis (Fig. 3*C*), DNA was restricted with *Eco*R I and hybridized with *probe III*, which abuts the *Eco*R I site at +1,299 bp. The *Eco*R I digestion liberated a 2.8-kb fragment that encompassed the promoter region, the first exon, and a portion of the first intron. With increasing DNase I digestion, a number of hypersensitive sites were identified that corroborated the sites identified with *probe II*, shown in Fig. 3*B*; an additional site within the first intron was also identified. The higher resolution of this analysis allowed more accurate mapping of these sites on the ET-1 gene.

A total of 16 kb of the ET-1 locus have been evaluated for DNase I-hypersensitive regions. A summary of the ET-1-hypersensitive sites identified using this method is given in Fig. 3*D*, with a number of hypersensitive sites found in the 5′-flanking region as well as within the gene. On the basis of previous experience with comparisons of hypersensitive sites to the location of protein-DNA interactions by in vivo footprinting, the hypersensitive sites are estimated to be within ±50 bp of the identified site. In both the low- and higher resolution studies, we observed no changes in the position of the hypersensitive sites under both control and thrombin-stimulated conditions. Figure 3, *A–C*, suggests a difference in the sensitivity to DNase I between control and thrombin-stimulated conditions; however, we observed contrasting results in the low- versus high-resolution studies. Specifically, it appears in Fig. 3*A* that the hypersensitive site in the promoter is more sensitive to an equivalent concentration of DNase I in thrombin-treated cells. On the other hand, when the site is displayed using a more proximal probe (Fig. 3*B*) or a higher resolution gel (Fig. 3*C*), the hypersensitive subsites in the promoter appear more resistant to DNase I in the thrombin-treated cells. Unfortunately, neither of these two trends was universally reproducible. At this point, we can only conclude that we do not observe a consistent difference in chromatin structure between control and thrombin-treated cells, a situation that may be addressed in the future with in vivo footprinting methodology.

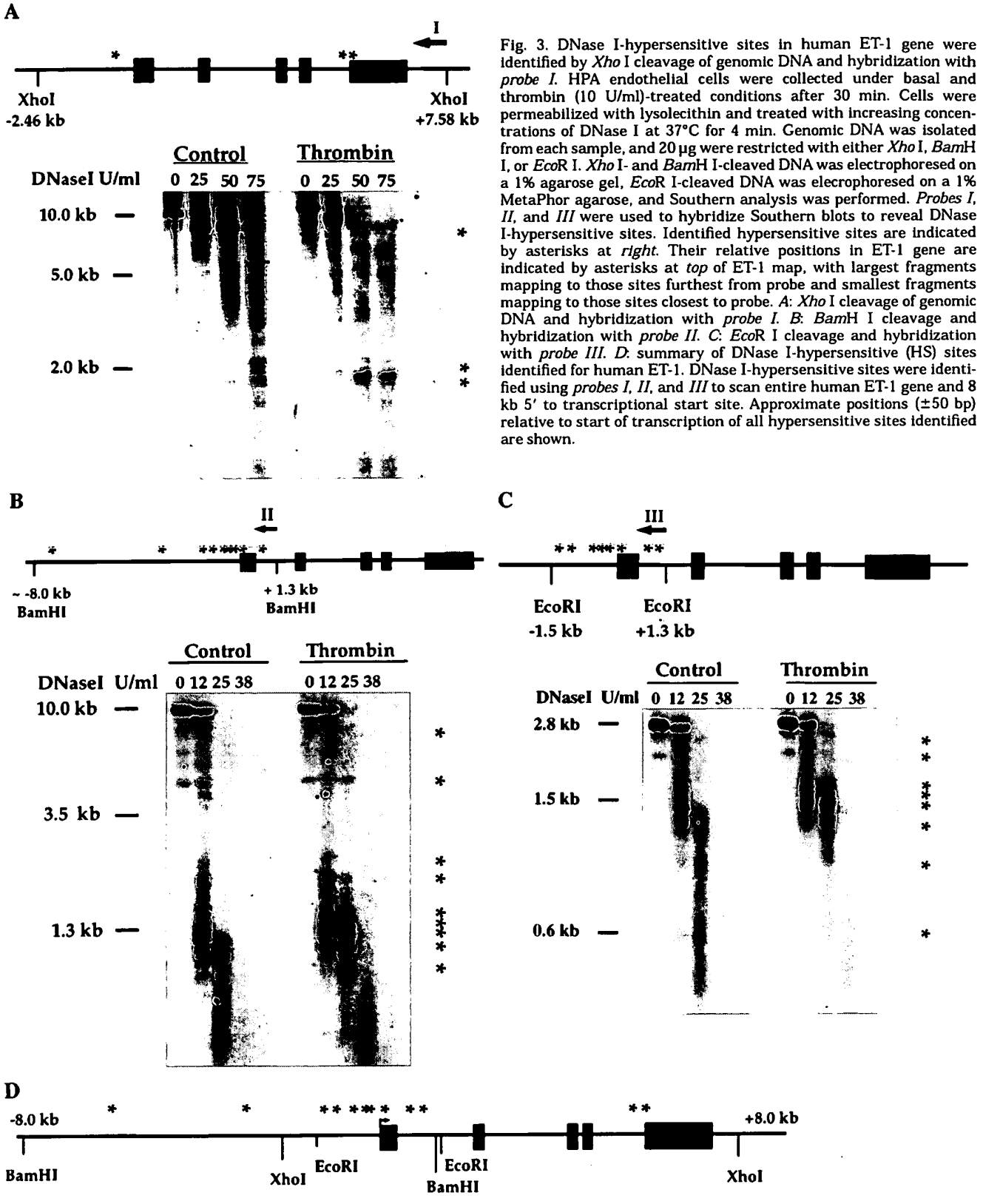

Fig. 3. DNase I-hypersensitive sites in human ET-1 gene were identified by *Xho* I cleavage of genomic DNA and hybridization with *probe I*. HPA endothelial cells were collected under basal and thrombin (10 U/ml)-treated conditions after 30 min. Cells were permeabilized with lysolecithin and treated with increasing concentrations of DNase I at 37°C for 4 min. Genomic DNA was isolated from each sample, and 20 µg were restricted with either *Xho* I, *Bam*H I, or *Eco*R I. *Xho* I- and *Bam*H I-cleaved DNA was electrophoresed on a 1% agarose gel, *Eco*R I-cleaved DNA was elecrophoresed on a 1% MetaPhor agarose, and Southern analysis was performed. *Probes I, II,* and *III* were used to hybridize Southern blots to reveal DNase I-hypersensitive sites. Identified hypersensitive sites are indicated by asterisks at *right*. Their relative positions in ET-1 gene are indicated by asterisks at *top* of ET-1 map, with largest fragments mapping to those sites furthest from probe and smallest fragments mapping to those sites closest to probe. *A*: *Xho* I cleavage of genomic DNA and hybridization with *probe I*. *B*: *Bam*H I cleavage and hybridization with *probe II*. *C*: *Eco*R I cleavage and hybridization with *probe III*. *D*: summary of DNase I-hypersensitive (HS) sites identified for human ET-1. DNase I-hypersensitive sites were identified using *probes I, II,* and *III* to scan entire human ET-1 gene and 8 kb 5′ to transcriptional start site. Approximate positions (±50 bp) relative to start of transcription of all hypersensitive sites identified are shown.

Promoter Region HS sites:	Within Gene HS sites:
-6.7 kb	+40 bp
-3.2 kb	+300 bp
-1.3 kb	+700 bp
-700 bp	+5.6 kb
-350 bp	+5.8 kb
-170 bp	
-150 bp	

Deletion analysis of the ET-1 promoter. To test the functionality of the putative *cis*-regulatory regions identified by chromatin structure analysis, plasmids containing various 5′-flanking sequences of the ET-1-promoter region were cloned into the hGH gene reporter vector pØGH. A linear map of each of the ET-1-promoter deletion fragments relative to the ET-1 gene is shown in Fig. 4*A*. As indicated, these fragments range in size from −8.0 kb to −88 bp of the 5′-flanking region, and all have a common *Bgl* II site at the 3′-end that maps to position +166 bp within exon 1. These constructs were transiently transfected into HPA and microvascular endothelial cells utilizing a combination of techniques that include DEAE-dextran with NuSerum, chloroquine diphosphate, and DMSO to improve transfection efficiency.

In lieu of cotransfection procedures to address transfection efficiency and variation in plasmid expression, we have used a batch transfection protocol, multiple independent preparations of plasmid DNA, Southern analysis of transfected plasmids, and a statistical analysis of a large number of independent experiments. The batch protocol allows for identical transfection efficiency from plate to plate for comparison of basal to thrombin-stimulated conditions within each experiment. The use of multiple preparations of plasmid and a statistical analysis of a large number of independent experiments has allowed for direct comparison of different experiments and plasmid constructs. Southern analysis of the plasmid from each plate from the original batch of transfected cells showed virtually identical plasmid levels, thereby indicating the same transfection efficiency in each plate from the original large batch of transfected cells.

Results of these experiments are shown in Fig. 4, *B* and *C*, for HPA and HMV endothelial cells, respectively. The average of between four and nine trials for each construct is illustrated. The reporter protein hGH is expressed in nanograms per milliliter of culture medium. For both cell lines studied, there were no detectable levels of hGH for the two smallest fragments, −88 and −141 bp. Some hGH protein was detected in the media, with transfection of the −378-bp fragment, but this level was submaximal in comparison with the larger constructs. A significant difference between control and thrombin-stimulated conditions was first detected with transfection of the −484-bp fragment, but, again, expression was not maximal. Maximal expression for both basal and thrombin-stimulated conditions was first seen with the −952-bp fragment. With increasing promoter deletion fragment sizes, there was no further increase in promoter activity as assessed by reporter protein secretion. The results were similar for both cell lines; however, the absolute level of hGH secreted was approximately twofold higher for the HMV cells in comparison with HPA cells.

These results indicate that a region between −141 and −378 bp is required for minimal ET-1 basal expression; however, this is not sufficient for maximal expression. Additionally, another element required for the thrombin-stimulated response is present between −378 and −484 bp. Each of these regions likely acts in concert with regions further upstream, between −484 and −952 bp, to confer full expression under both basal and stimulated conditions.

DISCUSSION

This study is the first detailed functional analysis of the human ET-1 promoter under both basal and thrombin-stimulated conditions. Previous studies of ET-1 gene regulation involving an endothelial cell model system relied on animal-derived or human umbilical vein endothelial cells (6, 11, 14, 15, 17–20, 23, 34, 36). However, it is possible that animal and umbilical vein endothelial cell-based models do not accurately reflect ET-1 regulation in human systemic or pulmonary vascular endothelial cells. Numerous examples provide evidence of differential gene regulation in endothelial cells derived from different tissues and species (2, 3, 7, 29). In addition, there are a limited number of reports detailing stimulus-dependent ET-1 gene regulation. These reports indicate an increase in ET-1-promoter activity after stimulation with insulin (23), thrombin, interleukin-1, or transforming growth factor-β (19); however, these analyses were cursory in that single constructs of 4.4 kb (23) and 2.9 kb (19) of the ET-1-promoter region were the only promoter fragments tested. Furthermore, these studies on the human promoter were performed in bovine aortic endothelial cells.

Thrombin plays an important role in a variety of vascular pathophysiologies, including pulmonary hypertension and acute lung injury (9, 13). Thrombin also has been shown to induce ET-1 expression (11, 19, 36), thus potentially elevating the levels of this potent vasoconstrictor in a variety of vascular diseases. Therefore, we evaluated thrombin-dependent regulation of ET-1 in both HPA and HMV endothelial cells. On the basis of the potency of ET-1, small alterations in ET-1 levels can cause dramatic changes in vascular tone. We therefore feel that understanding the increases in ET-1 expression will provide important physiological information.

In conjunction with our functional studies on the promoter, we have examined the chromatin structure of the ET-1 locus via DNase I treatment of lysolecithin-permeabilized cells. This in situ (24) method of analysis allows the cells to remain intact during DNase I treatment and has been shown to display very efficient DNA replication and transcription activities (5, 21). We observed numerous DNase I-hypersensitive regions in the ET-1 locus, with the majority of these regions concentrated in the 5′-promoter region of the gene. We found no detectable difference in sensitivity to DNase I in basal versus thrombin-stimulated cells, suggesting that the ET-1 gene promoter is a preset promoter in which the promoter is poised for transcriptional activation (12, 33) rather than having an inducible hypersensitive site that would be associated with remodeling promoters.

The functional significance of these hypersensitive sites was established for both basal and thrombin stimulation of human pulmonary endothelial cells.

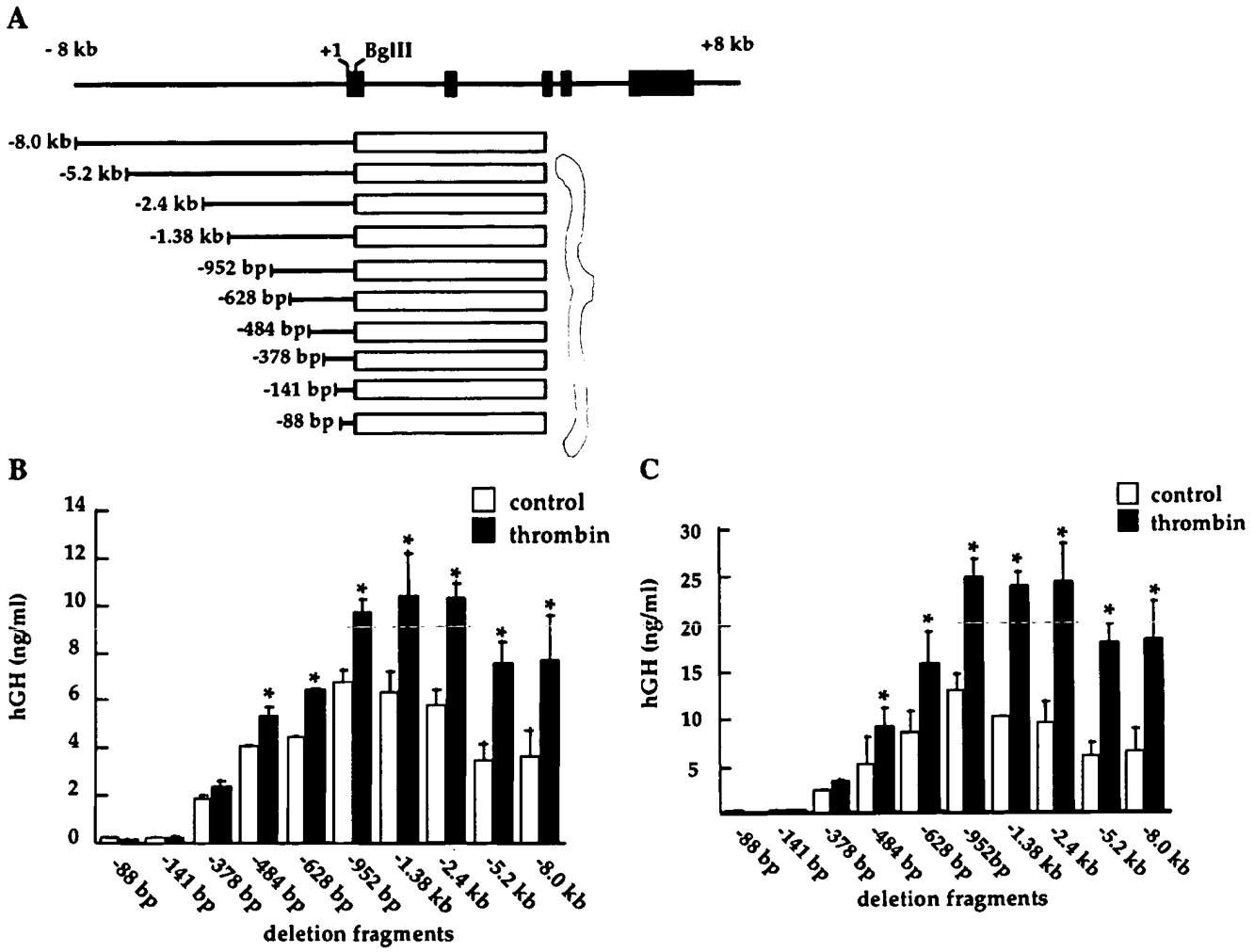

Fig. 4. 5′-Promoter deletion fragments of ET-1 promoter cloned into pØGH reporter vector and functional analysis of these fragments transiently transfected into HPA and HMV endothelial cells. *A*: a linear map of ET-1 gene is illustrated at *top*. Below this is a linear representation of regions of human ET-1 gene used to construct each promoter deletion construct. Each of the ET-1-promoter deletion fragments has a common *Bgl* II site at the 3′-end that lies +166 bp relative to start of transcription (+1) and is cloned into a compatible *Bam*H I site within polylinker region of pØGH (open bars). *B* and *C*: levels of hGH protein measured in media from HPA (*B*) and HMV (*C*) endothelial cells transiently transfected with various ET-1-promoter deletion fragments fused to an hGH expression vector. Data are means ± SE of 4–9 individual experiments for each construct. * Significant difference between control and thrombin-stimulated values for each vector ($P < 0.05$).

These analyses demonstrate that an important element for basal regulation of ET-1 lies between −378 and −141 bp. Furthermore, thrombin-stimulated induction minimally required an additional element between −484 and −378 bp. However, neither of these putative elements was sufficient for full expression of the reporter gene. There was a gradual increase in reporter expression, with increasing sizes of promoter fragment transfected, under both basal and thrombin-stimulated conditions. Full expression was apparent for both conditions with the −952-bp fragment, and larger fragments showed no additional increases in reporter expression.

A composite of the estimated regions of the DNase I-hypersensitive sites in the ET-1 gene and 5′-flanking region deletion analysis are shown in Fig. 5. Only those constructs that contain regions of DNase I hypersensitivity with an increase in construct size are shown. The putative *cis*-regulatory regions closely defined by DNase I correlate with those regions shown to be important for basal and thrombin-stimulated expression of ET-1 in transient transfection assays. It should be noted that no hypersensitive sites are detected when basal expression is lost (both the −88- and −141-bp constructs). The detection of the first three hypersensitive regions directly correlates with basal expression found in the −378-bp construct. Thrombin-inducible expression is first detected with the −484-bp construct, which does not correlate with any additional region of hypersensitivity. However, it might be hypothesized that the 5′ most hypersensitive site contained within the −378-bp

construct may in fact be responsible for thrombin-inducible expression of ET-1. This site may lie too close to the boundary of the −378-bp construct, thereby interfering with its function and not allowing the −378-bp construct to confer thrombin-stimulated expression. Also, the hypersensitive sites are estimated based on the size of the fragments and size markers on the gel, with some margin of error. Full basal and thrombin-stimulated reporter gene expression is only detected with the −952-bp construct, which contains the next hypersensitive site at approximately −700 bp, suggesting that this site may be responsible and necessary for conferring full basal and thrombin-stimulated expression of the ET-1 promoter. Although additional hypersensitive sites become apparent further upstream, these do not seem to have an effect on reporter gene expression. These sites may be constitutive sites that are important for other stimuli.

The number of hypersensitive regions and the increasing level of expression with both basal and thrombin stimulation demonstrated in our deletion analysis of the ET-1-promoter region suggest a complex regulatory mechanism. We believe that several interacting regions with a number of possible *cis*-acting elements cooperate to allow complete basal and stimulated transcription. With the requirement of a number of interacting regulatory regions for full basal and regulated expression and thus the lack of a clearly defined unique regulatory site, further deletion analyses will not provide additional information. Generation of constructs using partial fragments of the ET-1-promoter region will result in the loss of integrity of the ET-1 promoter and may interrupt the interactions of these regulatory sites. In fact, we have inserted a region of the ET-1 promoter from −2.4 kb to −500 bp in a minimal thymidine kinase promoter-hGH plasmid that resulted in no reporter expression (Golden, unpublished data). To further identify the specific regions involved, higher resolution studies that still maintain the integrity of the gene are necessary. This can be accomplished by in vivo footprinting of the ET-1 gene promoter region under basal and thrombin stimulation to identify important protein-DNA contacts from −952 to −141 bp.

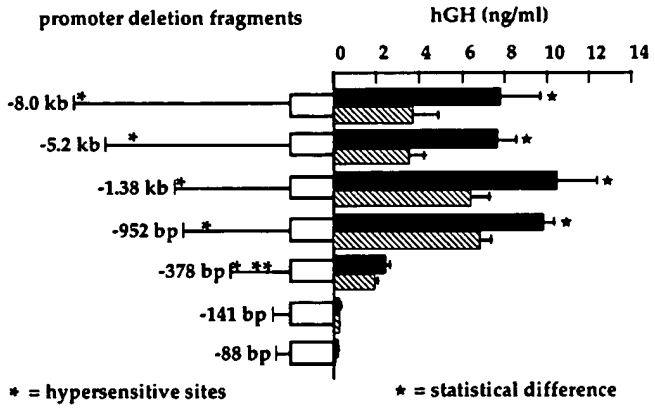

Fig. 5. Schematic comparison of functional analysis of ET-1-promoter deletion fragments and positions of DNase I-hypersensitive sites. *Left*: some of ET-1-promoter deletion fragments used in transient transfection analysis. Two smallest fragments are shown, and thereafter, only those promoter deletion fragments containing new hypersensitive sites are illustrated. *Location of new DNase I-hypersensitive sites contained within each successively larger fragment. *Right*: data are identical to those shown in Fig. 4*B* and illustrate corresponding hGH reporter protein levels after transfection of HPA endothelial cells with each of the ET-1-promoter deletion fragments that contains new hypersensitive sites. *Significant difference between control and thrombin-stimulated values ($P < 0.05$).

Our results of ET-1 regulation differ from those described in heterologous systems, in which the human ET-1-promoter regions were transfected into bovine aortic endothelial cells (17, 34). We obtained −88- and −141-bp ET-1-hGH constructs that were previously used in evaluating basal expression in bovine cells (34). The −88-bp construct resulted in no basal expression in both human and bovine endothelial cells, and the −144-bp construct resulted in basal expression in bovine aortic endothelial cells but not in human pulmonary arterial endothelial cells. We have performed similar studies in human aortic endothelial cells and have found them to be similar to the human pulmonary endothelial cells (data not shown); therefore, the differences found between our study and the bovine aortic endothelial cell studies are not due to the difference between aortic and pulmonary endothelial cells. Also, our studies in human cells and the studies in bovine cells are remarkably different than what has been observed for basal human ET-1-promoter activity in COS cells (monkey kidney epithelial cells) (1). Variable results for basal human ET-1-promoter activity have also been observed in HeLa cells, with the −143- and −141-bp constructs of the promoter region having no activity and some basal activity, respectively (17, 34), and the −129-bp region generating no activity (1). In bovine aortic endothelial cells, the region between −141 and −127 bp of the ET-1 promoter is required for full basal transcriptional activity, and a protein-binding motif, essential for basal regulation, lies within this region between −136 and −131 bp (17, 34). Our results using a homologous system with the human promoter in human pulmonary endothelial cells demonstrate that additional regions aside from the −141-bp promoter region are required for ET-1-promoter activity. We observed no detectable level of basal expression by ET-1 fusion constructs when the size of the ET-1-promoter fragment was −141 bp or smaller, and this is corroborated by the fact that there are no resident hypersensitive sites within this region of the gene. On the basis of our results, we believe that the initial basal expression requires a region between −378 and −141 bp, whereas full expression is not apparent until a much larger fragment, −952 bp, is transfected.

Computer analysis of the ET-1-promoter region has identified a number of elements that may have a regulatory role. For example, several activator protein-1-like consensus sequences and a nuclear factor-κB sequence have been identified. However, not all of these sites are located near the hypersensitive sites identified or in the region demonstrating functional activity. In addition, Scarpati and DiCorleto (28) previously identified a thrombin response element (CCACCCACC); how-

ever, we were unable to detect this element in the sequenced region of the ET-1 promoter that includes the thrombin-dependent regulation, indicating that we have detected a novel thrombin *cis*-acting element.

Our studies have provided an analysis of the transcriptional activity of the ET-1 promoter. These data are the first such analyses of the function of the human ET-1 promoter in a homologous system using a tissue type relevant to human disease, specifically, pulmonary disease. It is also the first detailed analysis of stimulus-induced ET-1-promoter function. At present, the transcriptional regulation of the ET-1 gene appears to be a complex system, with the possibility of a number of interacting regulatory sites involved in full basal and thrombin-dependent expression. This may not be surprising, since ET-1 is an extremely potent vasoconstrictor and its expression must be highly regulated to ensure the maintenance of appropriate vascular tone.

We thank J. Philip Kohler and Sandra Fogg for technical support on this project and Dr. David Wilson for kindly providing a number of the ET-1-hGH constructs.

This study was supported by an American Heart Association of Florida Grant-in-Aid and a National Aeronautics and Space Administration Space Grant Fellow Award to C. L. Golden.

Address for reprint requests: G. A. Visner, 1600 SW Archer Rd., Rm. D2-15, PO Box 100296, Dept. of Pediatrics, Univ. of Florida, Gainesville, FL 32610.

Received 3 April 1997; accepted in final form 12 February 1998.

REFERENCES

1. **Benatti, L., L. Bonecchi, L. Cozzi, and P. Sarmientos.** Two preproendothelin 1 mRNAs transcribed by alternative promoters. *J. Clin. Invest.* 91: 1149–1156, 1993.
2. **Caveda, L., M. Corada, I. Martin-Padura, A. Del Maschio, F. Breviario, M. G. Lampugnani, and E. Dejana.** Structural characteristics and functional role of endothelial cell to cell junctions. *Endothelium* 2: 1–10, 1994.
3. **Chen, X., S. E. Orfanos, J. W. Ryan, A. Y. K. Chung, D. C. Hess, and J. D. Catravas.** Species variation in pulmonary endothelial aminopeptidase P activity. *J. Pharmacol. Exp. Ther.* 259: 1301–1307, 1991.
4. **Chomczynski, P., and N. Sacchi.** Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162: 156–159, 1987.
5. **Contreras, R., and W. Fiers.** Initiation of transcription by RNA polymerase II in permeable, SV40-infected or noninfected, CV1 cells; evidence for multiple promoters of SV40 late transcription. *Nucleic Acids Res.* 9: 215–236, 1981.
6. **Dorfman, D. M., D. B. Wilson, A. P. Bruns, and S. H. Orkin.** Human transcription factor GATA-2. Evidence for regulation of preproendothelin-1 gene expression in endothelial cells. *J. Biol. Chem.* 267: 1279–1285, 1992.
7. **Draijer, R., A. B. Vandrager, C. Nolte, H. R. de Jonge, U. Walter, and V. W. van Hinsbergh.** Expression of cGMP-dependent protein kinase I and phosphorylation of its substrate, vasodilator-stimulated phosphoprotein, in human endothelial cell of different origin. *Circ. Res.* 77: 897–905, 1995.
8. **Druml, W., H. Stelzner, W. Waldhausl, K. Lenz, A. Hammerle, H. Vierhapper, S. Gasic, and O. F. Wagner.** Endothelin-1 in adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 148: 1169–1173, 1993.
9. **Eisenberg, P. R., C. Lucore, L. Kaufman, B. Sobel, A. S. Jaffe, and S. Rich.** Fibrinopeptide A levels indicative of pulmonary vascular thrombosis in patients with pulmonary hypertension. *Circulation* 82: 841–847, 1990.
10. **Giaid, A., M. Yanagisawa, D. Langleben, R. P. Michel, R. Levy, H. Shennib, S. Kimura, T. Masaki, W. P. Duguid, F. R. C. Path, and D. J. Stewart.** Expression of endothelin-1 in the lungs of patients with pulmonary hypertension. *N. Engl. J. Med.* 328: 1732–1739, 1993.
11. **Golden, C. L., J. P. Kohler, H. S. Nick, and G. A. Visner.** Effects of vasoactive and inflammatory mediators on endothelin-1 expression in pulmonary endothelial cells. *Am. J. Respir. Cell Mol. Biol.* 12: 503–512, 1995.
12. **Gross, D. S., and W. T. Garrard.** Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* 57: 159–197, 1988.
13. **Horgan, M. J., J. W. Fenton II, and A. B. Malik.** Thrombin-induced pulmonary vasoconstriction. *J. Appl. Physiol.* 63: 1993–2000, 1987.
14. **Kawana, M., M. Lee, E. E. Quertermous, and T. Quertermous.** Cooperative interaction of GATA-2 and AP1 regulates transcription of the endothelin-1 gene. *Mol. Cell. Biol.* 15: 4225–4231, 1995.
15. **Kourembanas, S., P. A. Marsden, L. P. McQuillan, and D. V. Faller.** Hypoxia induces endothelin gene expression and secretion in cultured human endothelium. *J. Clin. Invest.* 88: 1054–1057, 1991.
16. **Langleben, D., M. Demarchie, D. Laporta, A. H. Spanier, R. D. Schlesinger, and D. J. Stewart.** Endothelin-1 in acute lung injury and the adult respiratory distress syndrome. *Am. Rev. Respir. Dis.* 148: 1646–1650, 1993.
17. **Lee, M., K. D. Bloch, J. A. Clifford, and T. Quertermous.** Functional analysis of the endothelin-1 gene promoter. *J. Biol. Chem.* 265: 10446–10450, 1990.
18. **Lee, M., M. S. Dhadly, D. H. Temizer, J. A. Clifford, M. Yoshizumi, and T. Quertermous.** Regulation of the endothelin-1 gene expression by *fos* and *jun*. *J. Biol. Chem.* 266: 19034–19039, 1991.
19. **Maemura, K., H. Kurihara, T. Morita, Y. Oh-hashi, and Y. Yazaki.** Production of endothelin-1 in vascular endothelial cells is regulated by factors associated with vascular injury. *Gerontology* 8, *Suppl.* 1: 29–35, 1992.
20. **Marsden, P. A., and B. M. Brenner.** Transcriptional regulation of the endothelin-1 gene by TNF. *Am. J. Physiol.* 262 (*Cell Physiol.* 31): C854–C861, 1992.
21. **Miller, M. R., J. J. Castellot, and A. B. Pardee.** A permeable animal cell preparation for studying macromolecular synthesis. DNA synthesis and the role of deoxyribonucleotides in S phase initiation. *Biochemistry* 17: 1073–1080, 1978.
22. **Murphrey, J. M., and S. A. Chrysogelos.** Direct measurements of transfection efficiency in transient transfection. *Biotechniques* 18: 967–969, 1995.
23. **Oliver, F. J., G. de la Rubia, E. P. Feener, M. Lee, M. R. Loeken, T. Shiba, and T. Quertermous.** Stimulation of endothelin-1 gene expression by insulin in endothelial cells. *J. Biol. Chem.* 266: 23251–23256, 1991.
24. **Pfeifer, G. P., and A. D. Riggs.** Chromatin differences between active and inactive X chromosomes revealed by genomic footprinting of permeabilized cells using DNase I and ligation-mediated PCR. *Genes Dev.* 5: 1101–1113, 1991.
25. **Risau, W.** Differentiation of endothelium. *FASEB J.* 9: 926–933, 1995.
26. **Rubanyi, G. M.** *Endothelin.* New York: Oxford Univ. Press, 1992, p. 72–102.
27. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
28. **Scarpati, E. M., and P. E. DiCorleto.** Identification of a thrombin response element in the human platelet-derived growth factor B-chain (c-sis) promoter. *J. Biol. Chem.* 271: 3025–3032, 1996.
29. **Speiser, W., E. Anders, K. T. Preissner, O. Wagner, and G. Muller-Berghaus.** Differences in coagulant and fibrinolytic activities of cultured human endothelial cells derived from omental tissue microvessel and umbilical veins. *Blood* 69: 964–967, 1987.
30. **Stelzner, T. J., R. F. O'Brien, M. Yanagisawa, T. Sakurai, K. Sato, S. Webb, M. Zamora, I. F. McMurtry, and J. H. Fisher.** Increased lung endothelin-1 production in rats with idiopathic

pulmonary hypertension. *Am. J. Physiol.* 262 (*Lung Cell. Mol. Physiol.* 6): L614–L620, 1992.

31. **Stewart, D. J., R. D. Levy, P. Cerenacek, and D. Langleben.** Increased plasma endothelin-1 in pulmonary hypertension: marker or mediator of disease? *Ann. Intern. Med.* 114: 464–469, 1991.
32. **Visner, G. A., E. D. Staples, S. E. Chesrown, E. R. Block, D. S. Zander, and H. S. Nick.** Characterization and maintenance of human pulmonary artery endothelial cells in culture isolated from transplant donors. *Am. J. Physiol.* 267 (*Lung Cell. Mol. Physiol.* 11): L406–L413, 1994.
33. **Wallrath, L. L., Q. Lu, H. Granok, and S. C. R. Elgin.** Architectural variations of inducible eukaryotic promoters: preset and remodeling chromatin strutures. *Bioessays* 16: 165–170, 1994.
34. **Wilson, D. B., D. M. Dorfman, and S. H. Orkin.** A nonerythroid GATA-binding protein is required for function of the human preproendothelin-1 promoter in endothelial cells. *Mol. Cell. Biol.* 10: 4854–4862, 1990.
35. **Wu, C.** The 5′ ends of *Drosophila* heat shock genes in chromatin are hypersensitve to DNase I. *Nature* 286: 854–860, 1980.
36. **Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto, and T. Masaki.** A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 332: 411–415, 1988.